Spectrofluorimetry and Chemometrics for Investigation of Norfloxacin Distribution in Multilamellar Liposomes

N. L. ZALLOUM, D. N. BILOTI, and F. B. T. PESSINE*
Instituto de Química, UNICAMP, C.P. 6154, 13084-971 Campinas, S.P., Brazil

Norfloxacin (NFX), a fluoroquinolone, was encapsulated in multilamellar liposomes (MLV) of soy-bean phosphatidylcholine at pH 7.0. The observed affinity of this class of drugs for hydrophobic environments, such as phospholipid bilayers, could lead to a better understanding of the mechanism of uptake in bacteria. The fluorescent properties of NFX were examined both free in solution and in MLV, using anisotropy and fluorescence quenching measurements. The latter data was treated with a chemometric method to deconvolute the overlapped spectra of zwitterionic and neutral species of NFX in equilibrium at this pH. The results show that NFX incorporates into the lipidic bilayers with two different distributions of species: the zwitterionic form in the lipid/aqueous interface, and the neutral one, more towards the center of the bilayer.

Index Headings: **Norfloxacin; Liposomes; Fluorescence; Chemometrics.**

INTRODUCTION

Norfloxacin (NFX) is a partially hydrophobic fluoroquinolone antibiotic, which has a C-6 fluorine substituent and a piperazine ring at position 7.[1] These bactericidal agents yield activity against a broad spectrum of both Gram-positive and Gram-negative bacteria, inhibiting the bacterial gyrase enzyme (Topoisomerase II), and are highly effective in the treatment of a wide variety of infectious diseases, mainly in the urinary tract and, more recently, in the respiratory tract.[2–4] NFX was synthesized and registered in 1977 by Kyorin Pharmaceutical Co. (Japan) and was presented for therapeutic utilization in 1980 by Ito et al.[5] However, only in 1987 was Norfloxacin permitted to be used in the United States.[5]

An important feature of fluoroquinolones is their zwitterionic amphoteric nature. These molecules are ionized over the whole pH range due to the presence of both carboxylic and amino moieties (Fig. 1). These properties may influence the distribution of the drug and its affinity for lipid environments, particularly lipidic bilayers.[6] This characteristic is probably related to the phototoxic properties of NFX under solar ultraviolet (UV) radiation, such as induction of dermic tumors in rats, phototoxicity in mamarian cells *in vitro*, and[7–9] accumulation in lysosomes of HS68 human skin fibroblasts and other citoplasmatic organelles, since fluoroquinolones are good photosensitizers of reactive oxygen species.[10,11]

The entrapment of the fluoroquinolones in liposomes could be of therapeutic interest. In fact, liposomal devices might be capable of ensuring different pathways of interaction with microbial cells, compared to the normal routes followed by fluoroquinolones in entering cells. This behavior could be useful in the treatment of infections caused by quinolone-resistant bacteria or by microbes that are normally poorly sensitive to this class of drugs.[12] An investigation of the distribution of NFX in liposomes would also help clarify the interaction of this drug with biological membranes.

A liposomal formulation could increase drug concentration at the site of infection due to the increased uptake by the macrophages of the reticuloendotelial system and half-lifetime, in addition to decreased systemic absorption, minimizing the side effects such as phototoxicity.[13,14] In this way, these kinds of systems become more important owing to their capacity to induce macrophage functions and NO and cytokine production, which are necessary in microbian, antineoplasic, and AIDS therapies.

This paper describes some results on research involving NFX distribution in lipidic bilayers of multi-lamellar liposomes (MLV) at pH 7.0 using spectrofluorimetric measurements, such as fluorescence quenching and fluorescence anisotropy, combined with a chemometric method of analysis.

EXPERIMENTAL

Materials and Methods. *Preparation of Liposomes.* The multilamellar liposomes containing Norfloxacin (NFX-MLV) were prepared as follows. Soy-bean phosphatidylcholine (PC; Epikuron 200SH, 25 and 45 mg), cholesterol (15 mg), and NFX (10.0 μmol) were dissolved in a mixture of chloroform and methanol (2:1, v:v), and the solvents were removed by evaporation. The lipid film was hydrated with 10.0 mL of 0.010 mol/L HEPES buffer (pH 7.0) at 65 °C. The samples were centrifuged at 6000 rpm for 20 min, and the liposomal pellet was separated from the supernatant containing non-encapsulated drug. The liposomal pellet was resuspended in buffer, mixed, and re-centrifuged two additional times.

Anisotropy Measurements. Steady-state measurements of fluorescence anisotropy of free and liposomal NFX (pH 7.0) were obtained on a Jobin Yvon-Spex Spectrofluorimeter (Fluorolog 3), with an accessory having L geometry.

The theory of this technique is based on the excitation and emission of polarized light. The polarization is a result of the so-called photoselection of fluorophores, according to their orientation relative to the direction of the polarized excitation. Nevertheless, the emission can be depolarized by rotational diffusion, the most common cause, which implies an angular displacement during the lifetime of the excited state, in a way that the emitted radiation does not show the same orientation as the excitation one.

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* Author to whom correspondence should be sent. E-mail: fpessine@iqm.unicamp.br.

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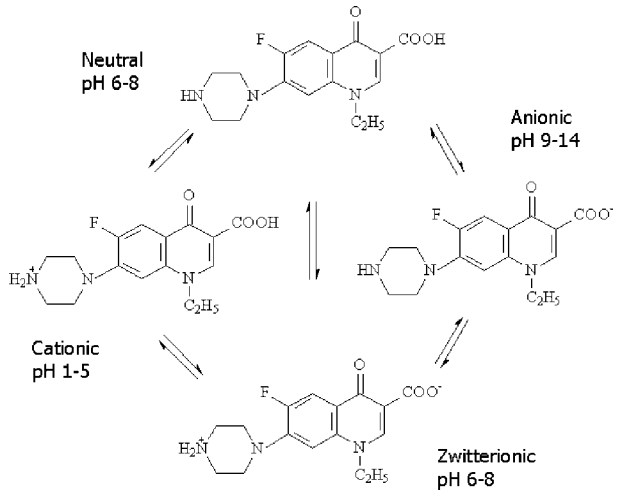

FIG. 1. Chemical equilibria of different species of NFX as a function of pH.

Equation 1 was used to calculate the degree of fluorescence anisotropy:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (1)$$

where $I_{\parallel}$ is the intensity measured through the polarizer oriented parallel to the direction of the polarized excitation, and $I_{\perp}$ is the intensity through the perpendicular direction. For a completely polarized light, $r = 1$.

Fluorescence Quenching Studies. Fluorescence quenching methods were applied to localize NFX in lipidic bilayers. All fluorimetric measurements were obtained on an SLM-AMINCO Spectrofluorimeter (SPF-500C), with a xenon lamp (250 W) as excitation source. The excitation wavelength was 284 nm, and fluorescence was monitored at 426 nm at a constant temperature. Free and encapsulated NFX were incubated in quartz cells at 25 °C (below the phase transition temperature of Epikuron, $T_m \approx 53$ °C) in the presence of iodide (I^-) and acrylamide as quenching agents in 0.010 mol·dm^{-3} HEPES buffer (pH 7.0). All solutions contained 0.010 mol·dm^{-3} sodium thiophosphate to prevent oxidation of the I^- ion, avoiding I_3^- formation.[15]

Data were analyzed according to the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_D[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher Q, respectively, and K_D is the Stern–Volmer collision constant.

To distinguish between two populations of fluorophores a treatment based on a modification of the Stern–Volmer equation was used:[16,17]

$$\frac{F_0}{\Delta F} = \left[\frac{1}{f_a K_D[Q]}\right] + \left(\frac{1}{f_a}\right) \quad (3)$$

where f_a is the fraction of the initial fluorescence that is accessible to the quencher Q, ΔF is the difference between the fluorescence intensity in the absence (F_0) and presence (F) of quencher, and K_D is the Stern–Volmer constant.

TABLE I. Fluorescence anisotropy values (*r*) for NFX and NFX-MLV, at 25 °C in several solvents.

Solutions of NFX (1.0×10^{-5} mol/L)	*r*
Deionized water	0.019
HEPES buffer (pH 7)	0.024
Citrate buffer (pH 3)	0.025
Ethanol	0.077
Chloroform	0.052
SDS (pH 7)	0.086
SDS (pH 3)	0.106
MLV (45 mg PC, NFX 1.0×10^{-4} mol/L)	0.378
MLV (25 mg PC, NFX 1.0×10^{-4} mol/L)	0.269

To resolve the overlapped spectra and quenching profiles of the zwitterionic and neutral forms, the chemometric method self-modeling curve resolution (SMCR), developed by Lawton and Sylvestre (1971), was applied in each experiment. This method uses a principal components analysis (PCA), based on kernel singular value decomposition (SVD).[18] In this case, it allows spectral deconvolution assuming the presence of only two substances. The SMCR method uses the following assumptions: (1) the curves must be non-negative; (2) the curves in the data set must be a linear combination of two linearly independent curves; and (3) at least one wavelength must exist for each substance where just that substances fluoresces.[19]

The SMCR method was carried out on a matrix constructed of relative fluorescence spectra, $F_0/\Delta F$.

RESULTS AND DISCUSSION

Anisotropy measurements were worthwhile as a strong indication of encapsulation. The results, listed in Table I, allowed the observation of values directly related to the kind of environment where the fluorophores were distributed. In hydrophobic environments, such as liposomal lipidic bilayers, higher values were noted, followed by values from micelles. This means that the molecules of the drug penetrate into these two systems, causing a difference in their rotational diffusion, indicating a more restricted degree of freedom in liposomal systems.

Free rotations in solution were related to the smallest values of *r*, compared to the states cited above. However, NFX showed a stronger interaction in ethanol and chloroform than in aqueous solutions, decreasing their rotational movements and increasing anisotropy values.

These results indicate that NFX molecules are inserted deeply into the lipidic bilayer of MLV liposomes, and the fluorescence quenching technique was used to obtain information on the localization of each specimen of NFX in the lipidic environment according to the pH to better understand the fluoroquinolone distribution in bacteria. In neutral medium, there is equilibrium between the neutral and zwitterionic forms of NFX, where the latter one predominates. It was expected that the neutral form would be incorporated into the bilayer, while the zwitterionic form would be in contact with the water/lipid interface.[6,20]

Fluorescence quenching studies were performed at pH 7 by using iodide and acrylamide as quenchers in free and liposomal NFX solutions. The concentrations of both quenchers were varied from 0 to 0.25 mol/L, a range in which it has been shown that quencher concentrations induce no changes in the lipid bilayer structure.[16]

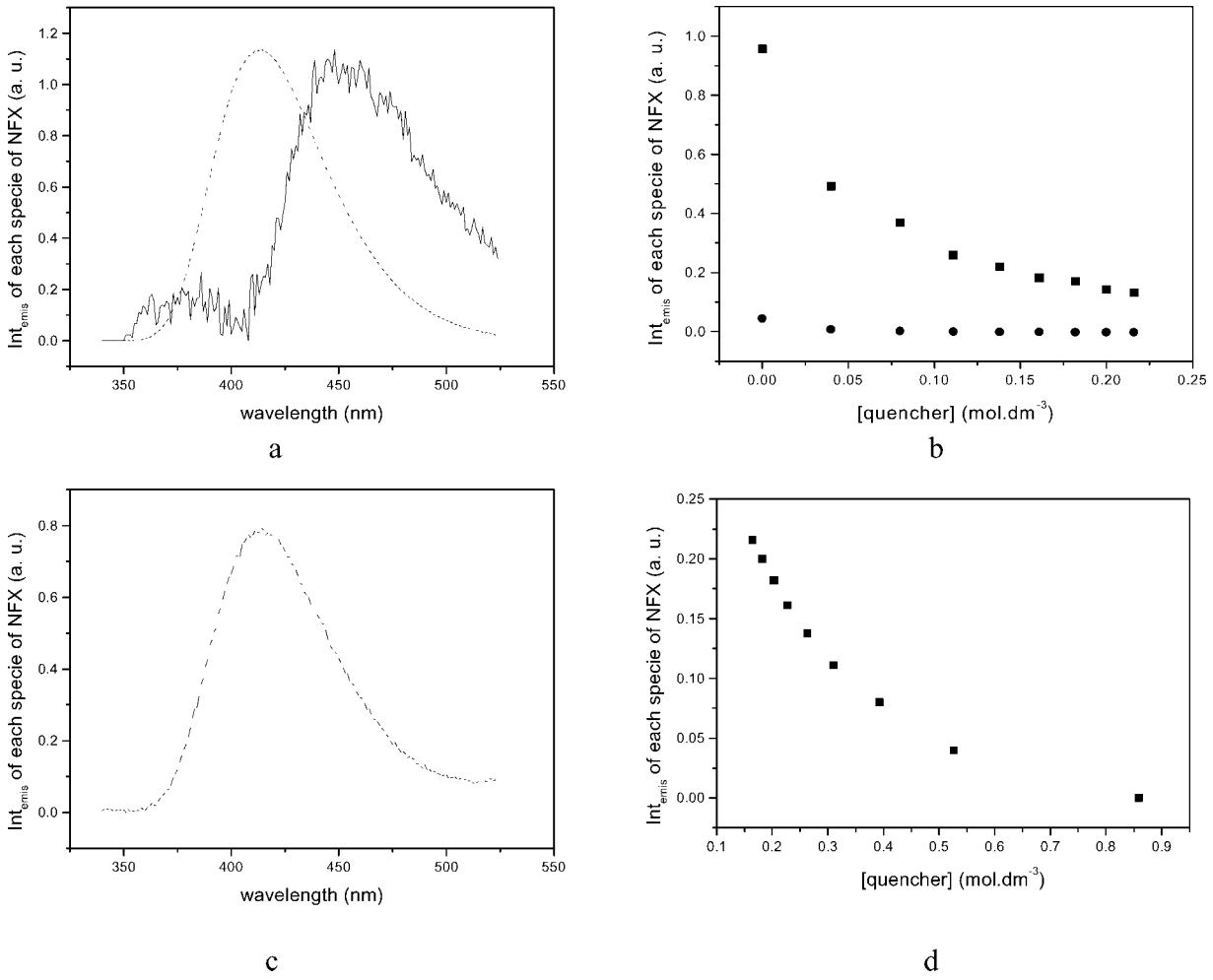

FIG. 2. (**a**, **c**) Resolved fluorescence spectra of zwitterionic (dashed line) and neutral forms (solid line) and (**b**, **d**) quenching fluorescence profiles of zwitterionic (squares) and neutral forms (circles), quenched by iodide in (**a**, **b**) the absence and (**c**, **d**) the presence of liposomes.

Applying the chemometric method developed by Lawton and Sylvestre[18] to the fluorescence spectral data, it was possible to obtain the resolved fluorescence spectra and the fluorescence quenching profiles of the neutral and zwitterionic forms of NFX separately (Figs. 2 and 3). However, the quenching constants were obtained from the experimental spectra and not from the calculated ones.

For each fluorescence quenching spectra the Stern–Volmer equation (Eq. 2) was applied, which describes the occurrence of dynamic and static quenching. The Stern–Volmer plots of these samples deviated from linearity with upward curvatures, suggesting the occurrence of both static and dynamic quenching (data not shown). Dynamic quenching is a process whereby external environmental influences (quenchers) interfere with the behavior of the excited state after its formation. It modifies both the emission intensity and the fluorescence lifetime. Otherwise, in static quenching, the quencher inhibits excited state formation, altering only the emission intensity.[21] In general, static and dynamic quenching can be distinguished by their different temperature dependences and viscosities, or, preferably, by lifetime measurements.

A linear Stern–Volmer plot is generally indicative of a single class of fluorophores, all equally accessible to the quencher.[17] The existence of two populations of fluorophores (neutral and zwitterionic forms) in liposomes at pH 7, which is in accordance with the partial hydrophobicity of NFX, allowed the use of the modified Stern–Volmer equation (Eq. 3) to obtain the rate of collisional encounters between the fluorophore and the quencher, called Stern–Volmer constant values, listed in Table II.

Iodide can quench the fluorescence of the drug only when the fluorophore is in hydrophilic regions. On the other hand, acrylamide is a hydrophobic molecule and can permeate into the bilayer. According to the modified Stern–Volmer constant values listed in Table II, it is seen that iodide quenches both forms of NFX when it is free in solution. However, in MLV liposomes, only the zwitterionic form was accessible to the quencher, although at a lower rate than that observed for the free form. The neutral species was not quenched by iodide because it was located deeper, near the center of the bilayer, since no polar molecules concentrate in the region that has the largest free volume.[20] This lack of quenching is interpreted as the inability of the charged and hydrated iodide to enter the nonpolar interior of the liposome, and also due to the low sensitivity of the equipment in detecting signals of small amounts of NFX inserted in the lipid bilayer. Therefore, one can conclude that the drug is incorporated in the MLV bilayers.

Utilizing acrylamide as quencher, it was observed that,

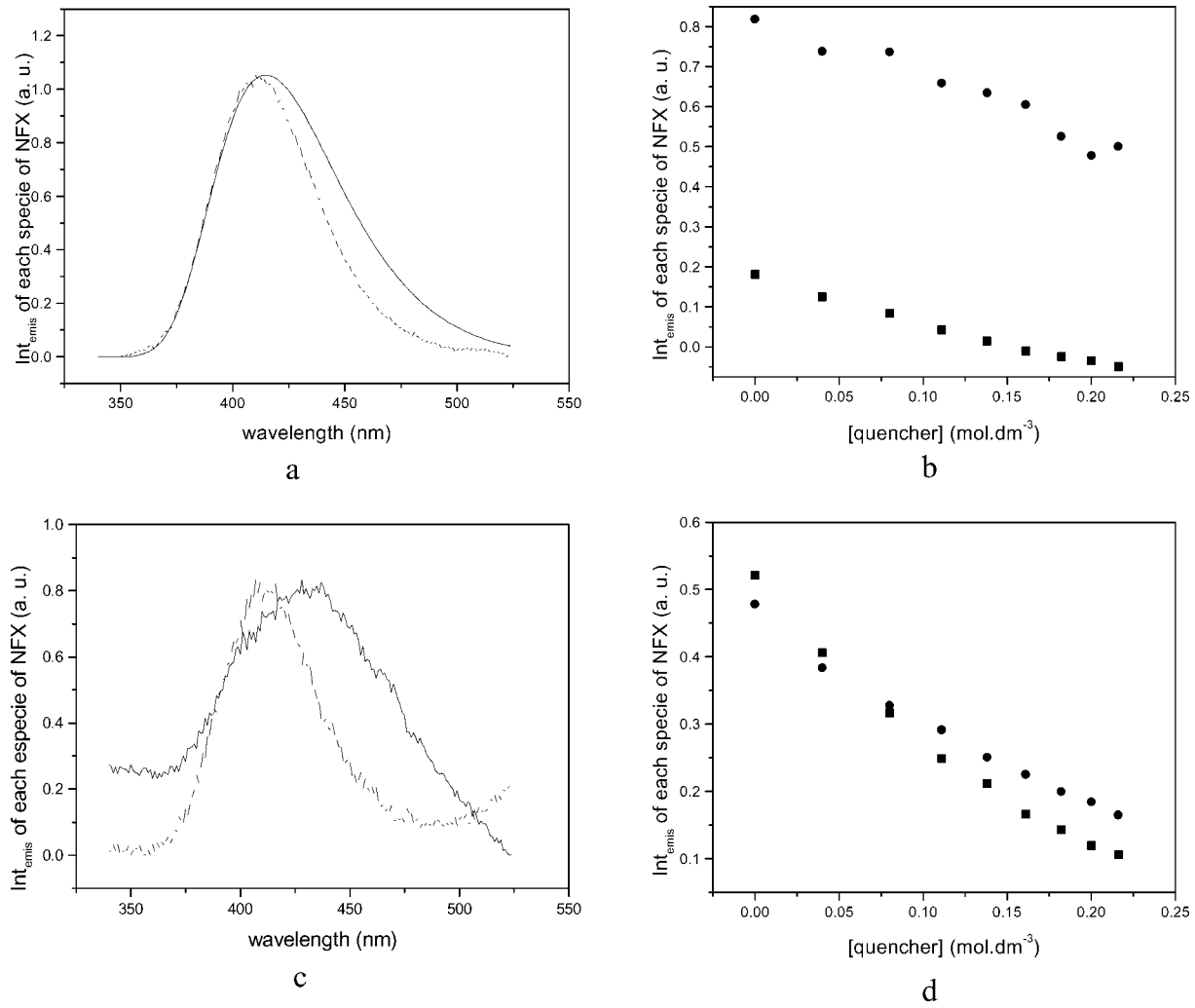

FIG. 3. (**a**, **c**) Resolved fluorescence spectra of zwitterionic (dashed line) and neutral forms (solid line) and (**b**, **d**) quenching fluorescence profiles of zwitterionic (squares) and neutral forms (circles), quenched by acrylamide in (**a**, **b**) the absence and (**c**, **d**) the presence of liposomes.

for the free drug in solution, the neutral form of NFX showed better interaction with this quencher than the zwitterionic form (K_{zwit} = 2.01 and K_{neut} = 3.32 L/mol). Also, a blue shift can be observed for the neutral specimen in the spectrum. Probably, the fundamental state of this species presents a smaller dipole moment than the excited state one. Such a state is better stabilized when interacting with acrylamide, which is a nonpolar molecule, as mentioned. This interaction leads to a shift to shorter wavelengths. In MLV liposomes, both zwitterionic and neutral forms were quenched by acrylamide, with the distribution analysis of each specimen being possible, confirming the existence of two populations of fluorophores (neutral and zwitterionic) in lipid bilayers of MLV liposomes.[22] The local insertion of acrylamide molecules in the lipid bilayer changes the environment around the fluorophore, increasing the interactions between the fluorophore and the quencher molecules. In this way there is an enlargement of the spectral emission for the neutral specimen, which is the one that has a better affinity for the acrylamide. The differences in the spectra recovered from acrylamide quenching measurements in water and lipid can also be attributed to the low sensitivity of the equipment. The descending behavior of quenching profiles proves the existence of such species, free in solution or inserted in the lipidic bilayers.

TABLE II. Stern–Volmer constants (K_D) obtained at pH 7.0 for free and encapsulated NFX using iodide and acrylamide as quenchers.

Sample	Quencher	NFX species	K_D (L/mol)
NFX	I^-	Zwitterion	71.8
		Neutral	21.8
NFX-MLV	I^-	Zwitterion	14.4
		Neutral	—
NFX	Acrylamide	Zwitterion	2.01
		Neutral	3.32
NFX-MLV	Acrylamide	Zwitterion	3.07
		Neutral	4.82

CONCLUSION

Anisotropy measurements give qualitative information on the incorporation of NFX molecules into the lipidic bilayers of liposomal vesicles. On the other hand, fluorescence quenching studies associated with chemometric methods reveal the existence of two predominate populations of NFX (neutral and zwitterionic forms) in multilamellar liposomes at pH 7.0.

The results showed that the zwitterionic form of NFX was incorporated into the lipid/aqueous interface of the liposomes, and the neutral form was located more toward the center of the bilayers. Iodide ions can quench the fluorescence of both forms of NFX in non-liposomal solution, but it cannot quench the encapsulated neutral form in the bilayer because these ions don't have access to the interior of the bilayers. On the other hand, acrylamide can be inserted into the bilayer, reaching the neutral species and therefore quenching its fluorescence emission. Monteiro et al.[6] suggested that both neutral and zwitterionic species permeate through the lipidic bilayers passively, although it is not possible to know their exact location. However, it should be emphasized that the neutral and zwitterionic spectra in lipidic bilayers could be better resolved, which was not observed due to the poor resolution of the chemometric method used.

This work suggests that only the neutral species reaches the interior of the bilayers, although both forms are present in the liposomes.

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